JIPB Journal of Integrative Plant Biology

Expression of the inactive ZmMEK1 induces salicylic acid accumulation and salicylic acid-dependent leaf senescence

Yuan Li, Ying Chang, Chongchong Zhao, Hailian Yang and Dongtao Ren*

State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China.
*Correspondence: ren@cau.edu.cn

Research Article

Abstract Leaf senescence is the final leaf developmental process that is regulated by both intracellular factors and environmental conditions. The mitogen-activated protein kinase (MAPK) signaling cascades have been shown to play important roles in regulating leaf senescence; however, the component(s) downstream of the MAPK cascades in regulating leaf senescence are not fully understood. Here we showed that the transcriptions of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* were induced during dark-induced maize leaf senescence. Furthermore, in-gel kinase analysis revealed the 42 kDa MAPK was activated. ZmMEK1 interacted with ZmSIMK1 in yeast and maize mesophyll protoplasts and ZmSIMK1 was activated by ZmMEK1 *in vitro*. Expression of a dominant negative mutant of *ZmMEK1* in *Arabidopsis* transgenic plants induced salicylic acid (SA) accumulation and SA-dependent leaf senescence. ZmMEK1 interacted with *Arabidopsis* MPK4 in yeast and activated MPK4 *in vitro*. SA treatment accelerated dark-induced maize leaf senescence. Moreover, blockage of MAPK signaling increased endogenous SA accumulation in maize leaves. These findings suggest that ZmMEK1-ZmSIMK1 cascade and its modulating SA levels play important roles in regulating leaf senescence.

Keywords: Leaf senescence; MAPK cascade; salicylic acid
Citation: Li Y, Chang Y, Zhao C, Yang H, Ren D (2016) Expression of the inactive ZmMEK1 induces salicylic acid accumulation and salicylic acid-dependent leaf senescence. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12465
Edited by: Qi Xie, Institute of Genetics and Developmental Biology, CAS, China
Received Aug. 17, 2015; **Accepted** Jan. 28, 2016
Available online on Jan. 29, 2016 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

Leaf senescence is the final step of the leaf development processes. The molecular mechanism of leaf senescence was extensively investigated in the model plant *Arabidopsis*. It has been established that leaf senescence is a highly regulated process (Guo et al. 2004; Lin and Wu 2004). During senescence, leaf cells undergo changes at the levels of structure, metabolism, and gene expression, including chloroplast breakdown, chlorophyll degradation, macromolecule hydrolysis, nutrient relocation from leaves to maturing seeds and energy metabolism alteration from carbon assimilation to catabolism (Lim et al. 2007; Guiboileau et al. 2010). These rearrangements are essential for seed formation, including biomass production and yield. Leaf senescence is thus regarded as a yield-limitation phase in crop plants (Gregersen et al. 2013; Thomas 2013). Global gene expression analysis revealed that thousands of genes were up-regulated in senescing *Arabidopsis* leaves. Among these genes, senescence-associated genes (SAGs) were showed to contribute greatly in controlling the onset and progression of leaf senescence (Buchanan-Wollaston et al. 2005; Breeze et al. 2011; Li et al. 2012b). A variety of transcription factors, belonging to WRKY, MYB, NAC, bZIP, C2H2-type zinc finger, and AP2/EREBP families, function as important regulators affecting senescence processes (Guo et al. 2004; Miao et al. 2004; Guo and Gan 2006; Guo and Gan 2011; Wu et al. 2012; Jiang et al. 2014).

Leaf senescence is an integrated event of leaf cells sensing and responding to internal and external signals. Environmental stresses, such as osmotic stress, extreme temperature, nutrient limitation, pathogen attacks, and light intensity and quality, have great influences in initiation and progression of leaf senescence (Lim et al. 2007). Hormones have been shown to play important roles in leaf senescence. Auxin and cytokinin act as negative regulators, while ethylene, salicylic acid, jasmonic acid, and abscisic acid act as positive regulators in leaf senescence regulation (Guo and Gan 2012; Jibran et al. 2013; Zhang et al. 2013). However, the molecular mechanisms of how these internal and external signals are transduced and integrated to finally influence leaf senescence need to be elucidated. To date, only a few components mediating signal transduction during leaf senescence have been identified. For example, soybean *SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE* (*GmSARK*) and its *Arabidopsis* homolog *AtSARK* are demonstrated to regulate leaf senescence through synergistic actions of auxin and ethylene (Xu et al. 2011), indicating that the receptor-like kinase(s) may function as key factor(s) in leaf senescence regulating signaling pathways.

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules in all eukaryotes. A typical MAPK cascade is composed of three protein kinases: MAPKKK (as MAP3K/MEKK), MAPKK (as MKK/MEK), and MAPK (as MPK), which sequentially phosphorylate and therefore activate in special ways to convey signals from upstream receptors to downstream targets (Ichimura et al. 2002). MAPK cascades are found in all studied plant species through the

analysis of genome sequences and activities of MAPKs response to external stimuli (Rodriguez et al. 2010). The *Arabidopsis* genome contains 60 MAPKKKs, 10 MAPKKs, and 20 MAPKs (Ichimura et al. 2002; Jonak et al. 2002). By sequence comparison and signature motif searches, orthologs of *Arabidopsis* MAPK genes in many other plant species, such as *Medicago truncatula*, tobacco, maize (*Zea mays*), and rice (*Oryza sativa*), have been identified and characterized (Rodriguez et al. 2010). Some MAPK members in plant species without genome sequence information were either cloned based on the partial protein sequence (as sucrose activated *CfSAPK* in *Cephalostachyum fuchsianum* Gamble) (Li et al. 2012a) or depended on homolog sequence information from other species (as *ZmMKK4* and *ZmMPK3* in maize) (Wang et al. 2010a; Kong et al. 2011).

MAPK cascades participate in numerous biological processes of plant growth and development (Rodriguez et al. 2010). Tobacco NPK1-NQK1-NRK1 cascade and *Arabidopsis* ANP1/2/3-MKK6-MPK4 cascade are known to regulate cytokinesis (Soyano et al. 2003; Kosetsu et al. 2010). YDA-MKK4/5-MPK3/6 pathway negatively regulates stomatal development in *Arabidopsis* (Pillitteri and Dong 2013). Recently it has been found that MKK9-MPK6 cascade plays a role in leaf senescence in *Arabidopsis* (Zhou et al. 2009). An in-gel kinase assay showed ZmMPK5 is activated in senescing leaves of maize (Berberich et al. 1999), implicating the involvement of MAPK pathways in leaf senescence in maize. However, the molecular mechanism of MAPK cascades modulating leaf senescence is not fully understood. In this article, we show that *ZmMEK1*, a maize MAPK kinase, is involved in leaf senescence. Biological analyses revealed ZmSIMK1 is a downstream MAPK of ZmMEK1. In transgenic *Arabidopsis* plants, expression of *ZmMEK1* dominant negative mutant (*ZmMEK1*^{KR}) leads to early leaf senescence. We further showed that endogenous salicylic acid (SA) accumulation induced by *ZmMEK1*^{KR} expression is required for the early leaf senescence. Exogenous SA treatment enhances dark-induced leaf senescence in maize, suggesting the positive role of SA in maize leaf senescence regulation. Our data indicate that this ZmMEK1-ZmSIMK1 cascade plays an important role in modulating leaf senescence.

RESULTS

Blockage of MAPK signaling accelerates dark-induced leaf senescence in maize

To explore whether MAPK signaling cascades are involved in the regulation of leaf senescence in maize, we first examined the rate of leaf senescence affected by PD98059, an inhibitor of MAP kinase kinase activity (Dudley et al. 1995). To obtain more synchronous and a better controlled process of senescence, we exploited the experimental system of individually darkened leaves (IDLs) developed by Weaver and Amasino (2001) which has been demonstrated to share many responses to natural and age-dependent senescence (Keech et al. 2007). Dark treatment was applied to the second leaves of 2-week-old maize seedlings. For 2 d in darkness, leaves turned yellow, which was a particular feature of senescence (Figure 1A). Senescence became more pronounced at 4 d (Figure 1A). This was qualified by the gradually reduced chlorophyll contents and increased ion leakage compared with untreated leaves (Figure 1B). We observed that the IDLs in the presence of PD98059 showed much more severe senescence phenotype than the control both at 2 and 4 d in darkness (Figure 1A). Also, at each time point, the chlorophyll contents were always lower and the membrane ion leakage was always higher in IDLs with PD98059 treatment than that in IDLs without PD98059 treatment (Figure 1B). These data indicated that treatment with PD98059 on IDLs accelerates the rate of senescence induced by darkness. Furthermore, the in-gel kinase assay showed a 42-kDa kinase is activated in the IDLs at 4 d in dark, and the activity of kinase disappeared in leaves treated with PD98059 and exhibited an accelerated senescence symptom (Figure 1C). Another 46-kDa kinase detected in all samples was considered as unspecific activation (Figure 1C). Inactivation of MAPKK-accelerated dark-induced leaf senescence suggested that a certain MAPK cascade may negatively regulate leaf senescence in maize plants.

To date, four maize MAPKK (*ZmMKK1*, -3, -4, and *ZmMEK1*) (Hardin and Wolniak 1998; Kong et al. 2011; Zhang et al. 2012; Cai et al. 2014) and seven MAPK (*ZmMPK3*, -4, -5, -6, -7, -17, *and ZmSIMK1*) (Berberich et al. 1999; Gu et al. 2010; Wang et al. 2010a; Pan et al. 2012) have been identified. Due to the difficulty in generating knock out mutants for all known MAPKKs and MAPKs, it is hard to rule out which MAPKK(s) and MAPK(s) mediated the leaf senescence. To explore the potential MAPKK(s) and MAPK(s), we then examined the transcript levels of the known MAPKK and MAPK genes in IDLs by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). As shown in Figure 1D, the transcript levels of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* displayed significant increase in leaves treated with dark for 1, 2, and 3 d, respectively, compared with control, and these increases were observed in all leaves with or without PD98059. *ZmMEK1* was gradually induced from 1 to 3 d of dark treatment. In the absence of PD98059, expressions of *ZmSIMK1* were dramatically increased at 0.5, 1, and 2 d of dark treatment and then reduced at 3 d, whereas *ZmSIMK1* displayed gradual increases from 0.5 to 3 d of dark treatment in the presence of PD98059. The change in the expression of *ZmMPK3* was similar to *ZmSIMK1*, except for no obvious alteration at 0.5 d (Figure 1D). In leaves with dark treatment for 4 d, the expression of all these genes exhibited dramatic reduction compared with control (<0.01-fold) (Figure 1D), possibly due to severe cell death in senescent leaves (Figure 1A, B). However, the transcript levels of the other three MAPKKs and five MAPKs did not show significant changes (<2.5-fold) in dark-induced senescing leaves either in the presence or absence of PD98059 (Figure S1). These results demonstrated that dark-induced leaf senescence promotes the transcription of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3*, implying that *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* might be the candidate members of MAPK in negatively regulating the leaf senescence process.

ZmSIMK1 is a downstream MAPK of ZmMEK1

To further investigate whether the ZmSIMK1 and ZmMPK3 are downstream MAPKs of ZmMEK1, we examined the interactions between ZmMEK1 and ZmMPK proteins using the yeast two-hybrid (Y2H) system. In the Y2H system, strong interaction between ZmMEK1 and ZmSIMK1 was observed,

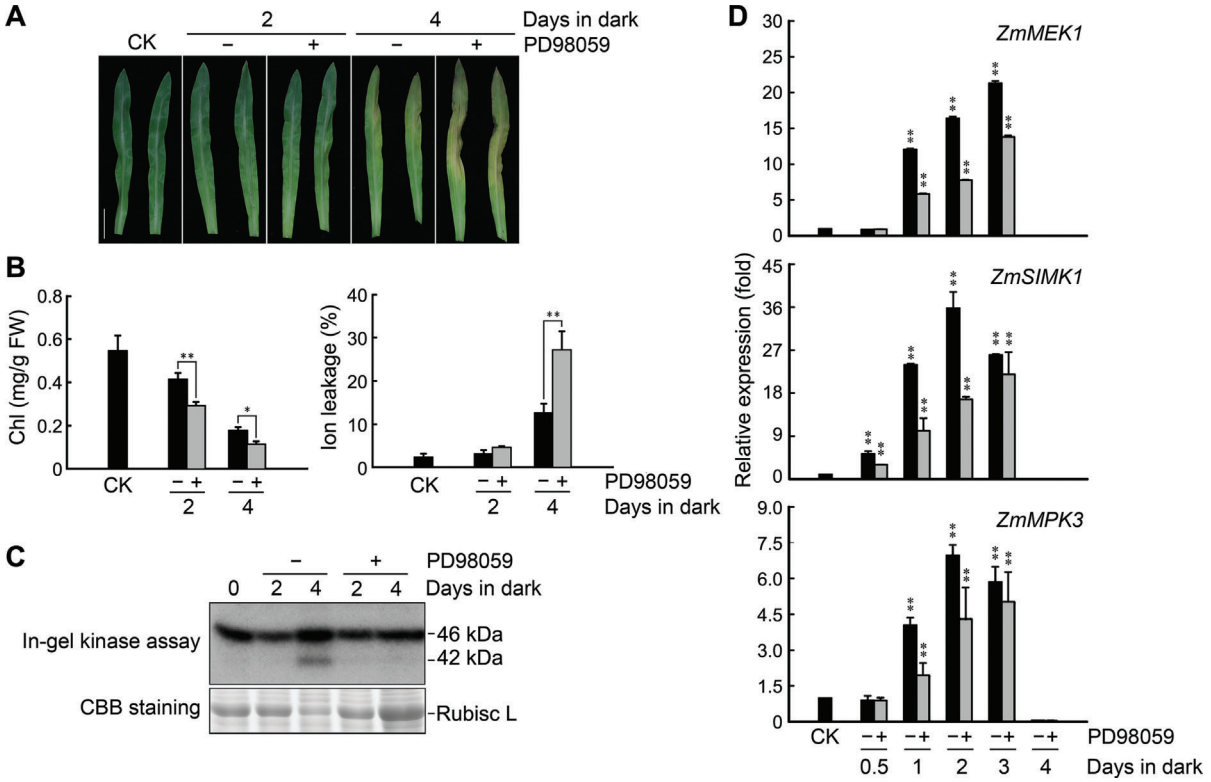

Figure 1. Inactivation of mitogen-activated protein kinase (MAPK) signaling accelerates dark-induced leaf senescence in maize plants, and transcript levels of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* increase in dark-induced senescent leaves

(**A**), (**B**) Effects of MAPKK inhibitor PD98059 on dark-induced leaf senescence. (**A**) The second leaves of 2-week-old maize seedlings were pretreated with 200 μmol/L PD98059 (+) or mock (−), and then covered with aluminium foil to induce senescence for 2 or 4 d, respectively. The second leaves of 2-week-old maize seedlings without treatment were used as a control (CK). Bar = 2 cm. (**B**) Chlorophyll (Chl) contents and membrane ion leakage in the leaves with indicated treatment. Three biological replicates were performed. Student's *t*-test analysis indicated the difference is significant ($^{*}P < 0.05$, $^{**}P < 0.01$). Error bars indicate *SD* from triplicate experiments. FW, fresh weight. (**C**) MAPK activities in the leaves with indicated treatment were detected by in-gel kinase assay using MBP as substrate (top). Image of Coomassie blue (CBB) staining was used as a loading control (bottom). (**D**) Expression of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* in leaves described in (**A**), as determined by quantitative real-time reverse transcription polymerase chain reaction. Representative fold changes normalized to the transcript levels of the control (CK) leaves discribed in (**A**). Error bars indicate *SD* from triplicate experiments, and asterisks indicate significant differences between the dark-induced senescent leaves and CK ($^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test).

while ZmMEK1 did not interact with other MAPKs, such as ZmMPK3, -4, -5, -6 -7, and -17 (Figure 2A). The interaction between ZmMEK1 and ZmSIMK1 was confirmed in planta by co-immunoprecipitation (CoIP) assay using maize mesophyll protoplasts. When 35S:Myc-ZmMEK1 and 35S:Flag-ZmSIMK1 fusion proteins were transiently co-expressed in maize mesophyll protoplasts, the Flag-ZmSIMK1 fusion protein, but not Flag-ZmMPK3 or -5, could be co-immunoprecipitated with Myc-ZmMEK1 (Figure 2B). These results suggested that ZmMEK1 interacts specifically with ZmSIMK1.

To determine whether ZmMEK1 can phosphorylate and activate ZmSIMK1, we performed *in vitro* kinase assays using FLAG-tagged recombinant ZmMEK1 and His-tagged ZmSIMK1, ZmMPK3, and ZmMPK5. For activation of MAPKKs, the phosphorylation of conserved serine (S) and/or threonine (T) residues located in the kinase activation loop is required. Substitution of these S/T residues with aspartic acid (D) or glutamic acid (G) has been used to mimic constitutively active forms of MAPKKs. Similarly, kinase inactive form of MAPKKs can be obtained by replacing a conserved lysine (K) residue for arginine (R) in the adenosine triphosphate (ATP)-binding domain. This approach of mutations triggering constitutive activation and inactivation of protein kinase has been a powerful strategy for functional studies of MAPKKs in plants (Ren et al. 2002; Takahashi et al. 2007; Liu et al. 2008). As such, we generated a constitutively active form of ZmMEK1 (as $ZmMEK1^{DD}$) by substituting the conserved S221/T227 in the activation loop with D/D and a constitutively inactivate form ($ZmMEK1^{KR}$) by mutating the conserved K100 of the ATP-binding site with R. The wild type ZmMEK1 is correspondingly named as $ZmMEK1^{WT}$. We expressed and purified FLAG-tagged $ZmMEK1^{WT}$, $ZmMEK1^{DD}$, and $ZmMEK1^{KR}$, and His-tagged ZmSIMK1, ZmMPK3, and ZmMPK5 proteins, from *Escherichia coli* to test whether ZmMEK1 activates ZmSIMK1 *in vitro*. Autophosphorylation assays showed that both $ZmMEK1^{WT}$ and $ZmMEK1^{DD}$ had autophosphorylation activity

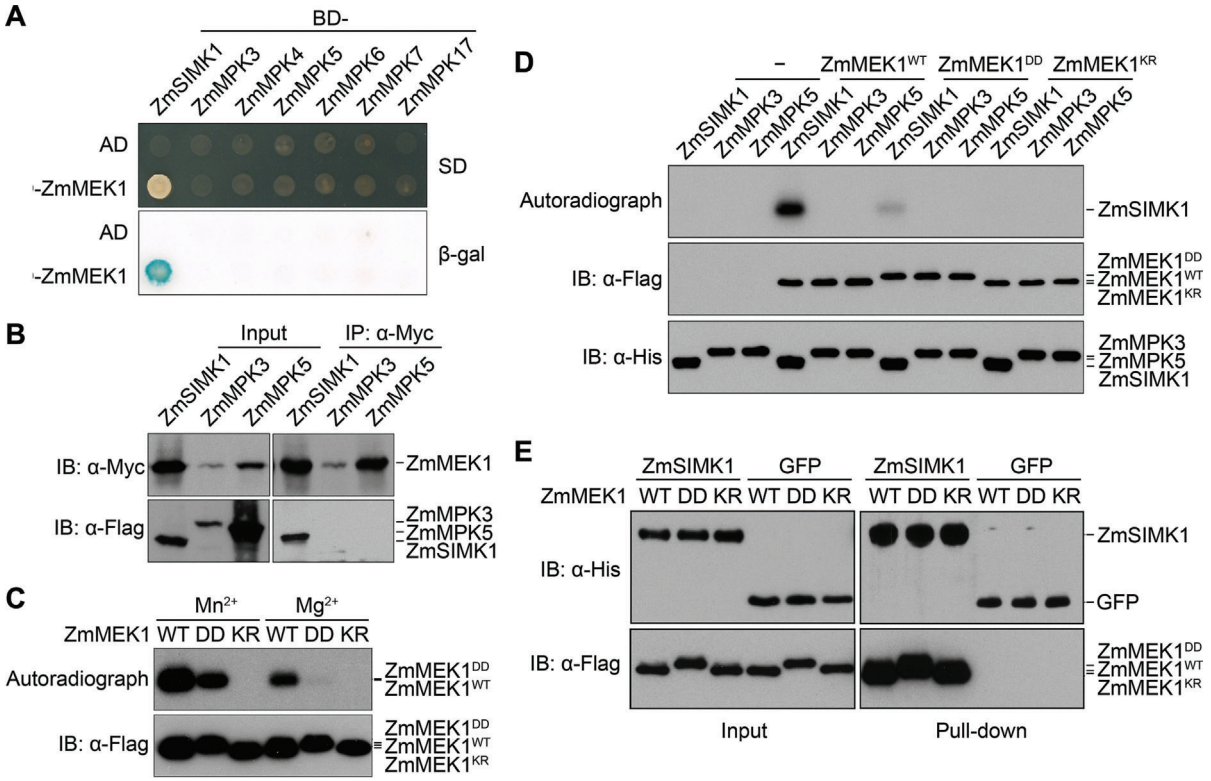

Figure 2. Interaction of ZmMEK1 and ZmSIMK1, and ZmMEK1 specifically phosphorylates ZmSIMK1

(**A**) Interaction between ZmMEK1 and ZmSIMK1 in the Y2H assay. ZmMEK1 fusions to GAL4 activating (AD) domain was coexpression with GAL4 binding domain fused ZmMPKs in yeast cells. Interaction was examined by yeast cell growth on synthetic defined (SD) medium lacking His, Leu, Trp, and Ade (upper panel), followed by assays for β-galactosidase activity (β-gal, bottom panel). (**B**) Coimmunoprecipitation of ZmMEK1 and ZmSIMK1 *in vivo*. Maize mesophyll protoplasts were transfected with *35S:Myc-ZmMEK1* and *35S:Flag-ZmSIMK1*/*35S: Flag-ZmMPK3*/*35S:Flag-ZmMPK5* as indicated. Total proteins extracted from protoplasts were immunoprecipitated with anti-Myc agarose beads. Total proteins (Input) and the immunoprecipitated proteins (right panel) were detected using anti-Myc antibody or anti-Flag antibody. (**C**) Autophosphorylation assay for recombinant ZmMEK1 proteins in the presence of Mn^{2+} or Mg^{2+}. Bacterially expressed Flag-tagged wild-type ZmMEK1 ($ZmMEK1^{WT}$), constitutively active variant ($ZmMEK1^{DD}$) and inactive variant ($ZmMEK1^{KR}$) were purified and used in *in vitro* kinase assays. Proteins were seperated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to X-ray film (top panel), and detected with anti-Flag antibody (bottom panel). (**D**) *In vitro* activation assay of ZmSIMK1 by ZmMEK1. Purified Flag-tagged ZmMEK1 recombinant proteins were incubated with (+) or without (−) His-tagged ZmSIMK1/ZmMPK3/ZmMPK5 as shown in a kinase reaction system for 30 min. Then the kinase activities of ZmSIMK1/ZmMPK3/ZmMPK5 were detected by an in-gel kinase assay using MBP as substrate and subjected to autoradiography (top panel). The protein amounts were indicated by the immunoblotting with anti-Flag (middle panel) and anti-His antibody (bottom panel). (**E**) Interactions between ZmMEK1 variants and ZmSIMK1 in pull-down assay. Recombinant His-tagged ZmSIMK1 was incubated with Flag-tagged $ZmMEK1^{WT}$, $ZmMEK1^{DD}$, and $ZmMEK1^{KR}$, respectively, in the kinase reation buffer for 30 min. His-tagged green fluorescent protein (GFP) was used as control. Total proteins were detected using anti-His antibody and anti-Flag antibody. Chelating Sepharose Fast Flow affinitied Ni^{2+} was used to pull down His-tagged ZmSIMK1 and GFP. ZmSIMK1 and GFP were detected with anti-His antibody and anti-Flag antibody was used to detect their interacting proteins (right panel).

in the presence of Mn^{2+} or Mg^{2+} ions, whereas, $ZmMEK1^{KR}$ had no detectable autophosphorylation activity (Figure 2C). Autophosphorylation activity of $ZmMEK1^{WT}$ suggested the ZmMEK1 belongs to the autoactive class of MAPKK (Kiegerl et al. 2000; Cardinale et al. 2002; Xu et al. 2008). We further detected the kinase activities of ZmSIMK1 using an in-gel kinase assay with Myelin basic protein (MBP) as substrate. As shown in Figure 2D, ZmSIMK1 exhibited kinase activities toward the substrate MBP in the presence of $ZmMEK1^{WT}$ and $ZmMEK1^{DD}$, while no kinase activities were detected for ZmMPK3 or ZmMPK5. The pull-down assays confirmed the physical interactions of ZmSIMK1 with $ZmMEK1^{WT}$, $ZmMEK1^{DD}$ or $ZmMEK1^{KR}$, respectively. $ZmMEK1^{WT}$, $ZmMEK1^{DD}$, and $ZmMEK1^{KR}$ could be detected in the pull-down samples of ZmSIMK1, but not in that of His-tagged green fluorescent protein (GFP) (Figure 2E). $ZmMEK1^{KR}$ also was pulled down by ZmSIMK1, indicating that the site mutation in ATP-binding domain of ZmMEK1 only abolished its kinase activity toward the downstream MAPK(s) but not the ability of interaction with its target(s). To our surprise, the kinase activity of

ZmSIMK1 activated by ZmMEK1^{DD} was lower than that by ZmMEK1^{WT}, which is not consist with several previously reported MAPKKs, such as MKK4, -5, -3, -9 in *Arabidopsis* and NtMEK2 in tobacco (Ren et al. 2002; Ren et al. 2008; Xu et al. 2014). The reason for this unexpected phenomenon is currently unknown.

Taking these results together, we suggest that ZmSIMK1 is a downstream MAPK of ZmMEK1.

Expression of the inactive ZmMEK1 in transgenic *Arabidopsis* plants induces early leaf senescence

In order to explore the possible function of *ZmMEK1*, we generated transgenic *Arabidopsis* lines expressing GFP-tagged *ZmMEK1*^{WT}, *ZmMEK1*^{DD}, and *ZmMEK1*^{KR} under the control of the Cauliflower mosaic virus 35S promoter. Two independent homozygous transgenic lines for each construct were used for later experiments. An empty vector transgenic line expressing GFP (*35S:GFP*) was created as a control. To validate the suitability of gene expression in *Arabidopsis*, we first confirmed the expression of all ZmMEK1 and GFP constructs by Western blotting. Bands of expected molecular weights were detected for all GFP fusion proteins (Figure S2). As shown in Figure 3A, *ZmMEK1*^{WT} and *ZmMEK1*^{DD} expressing lines showed no obvious phenotypes compared with the vector control, but the two *ZmMEK1*^{KR} expressing lines showed early leaf senescence phenotype, displaying dwarfism and early-yellowing rosette leaves, visible at 5 weeks after germination (Figure 3A). Consistently, leaves of both *ZmMEK1*^{KR} plant (5-week-old) retained half its chlorophyll contents and about two-fold of membrane ion leakage compared with that in *Vector*, *ZmMEK1*^{WT}, and *ZmMEK1*^{DD} plants (Figure 3B, C). Transcript levels of SAG12, a well-used senescence-associated gene, increased dramatically in *ZmMEK1*^{KR} plants (Figure 3D). These data demonstrated that expression of the inactive *ZmMEK1*^{KR} in *Arabidopsis* causes early leaf senescence. Notably, *ZmMEK1*^{KR} transgenic line 17 exhibited more severe phenotype of dwarfism and early-yellowing leaves than line 24 (Figure 3A). We also found that *ZmMEK1*^{KR} line 17 has a higher *ZmMEK1*^{KR} expression level than line 24 (Figure S2). This suggested a correlation between the ZmMEK1^{KR} protein level and the ZmMEK1^{KR}-induced leaf senescence.

Expression of *ZmMEK1*^{KR} promotes SA accumulation in transgenic plants

To elucidate the molecular mechanism of early senescence caused by the expression of *ZmMEK1*^{KR}, we determined the transcript levels of ethylene (ET-), jasmonic (JA-), and SA-responsive genes, respectively, in the *Vector*, *ZmMEK1*^{WT}, *ZmMEK1*^{DD}, and *ZmMEK1*^{KR} transgenic *Arabidopsis* plants. The transcript levels of SA-responsive genes, *PR1* and *PR5*, increased dramatically in *ZmMEK1*^{KR} plants, but no obvious change was detected in other transgenic plants (Figure 4A).

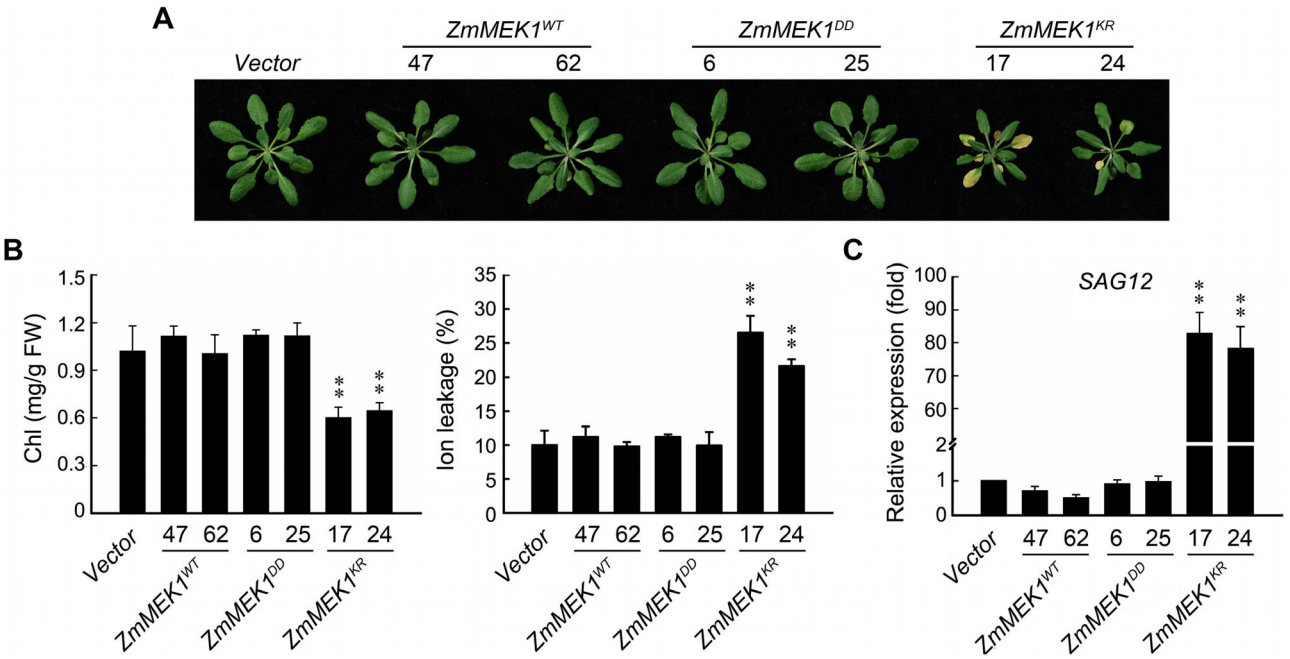

Figure 3. Inactive *ZmMEK1* causes early leaf senescence in transgenic *Arabidopsis*

(**A**) Phenotypes of the *ZmMEK1*^{WT}-, *ZmMEK1*^{DD}-, and *ZmMEK1*^{KR}-overexpressing and empty vector (*Vector*) plants. Plants were grown in soil under 16-h light/8-h dark growth conditions for 5 week and then photographed. Two independent transgenic lines of *ZmMEK1* were used for analysis. Bar = 1 cm. (**B**) Chlorophyll (Chl) contents and membrane ion leakage in leaves of *ZmMEK1*^{WT}, *ZmMEK1*^{DD}, *ZmMEK1*^{KR}, and *Vector* plants. The third pair of rosette leaves of individual *Arabidopsis* plants were detached and analyzed. (**C**) Quantitative real-time reverse transcription polymerase chain reaction analysis of *SAG12* transcript levels in leaves described in (**B**). Representative fold changes normalized to the transcript levels of leaves of *Vector* plant. The data in (**B**) and (**C**) are presented as the mean values of three biological replications. Error bars indicate *SD* from triplicate experiments, and asterisks indicate significant differences between the *ZmMEK1* transgenic *Arabidopsis* plants and *Vector* ($^{**}P < 0.01$, *t*-test).

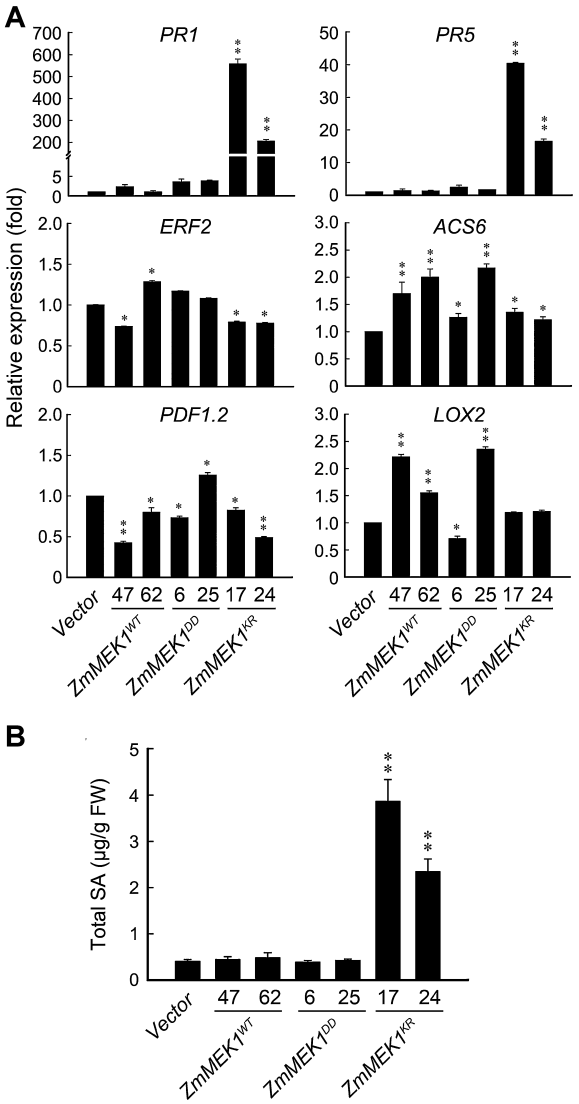

Figure 4. Accumulation of high-level SA in *ZmMEK1*^{KR} transgenic *Arabidopsis* plants

(**A**) Expression levels of responsive genes of SA (*PR1* and *PR5*), ethylene (*ERF2* and *ACS6*), and JA (*PDF1.2* and *LOX2*) in leaves of *ZmMEK1*^{KR} transgenic *Arabidopsis* plants. The third pair leaves of 5-week-old *Vector*, *ZmMEK1*^{WT}, *ZmMEK1*^{DD}, and *ZmMEK1*^{KR} plants were subjected to analysis. Expression levels were normalized to the transcript levels of *Vector* plants. (**B**) Total SA levels in the leaves decribed in (**A**) of *Vector*, *ZmMEK1*^{WT}, *ZmMEK1*^{DD}, and *ZmMEK1*^{KR} transgenic *Arabidopsis* plants. The data are presented as the mean values of three biological replications. Error bars indicate *SD* from triplicate experiments, and asterisks indicate significant differences between the *ZmMEK1* transgenic *Arabidopsis* plants and *Vector* ($^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test).

The transcript levels of ET-responsive genes (as *ERF2* and *ACS6*) and JA-responsive genes (as *PDF1.2* and *LOX2*) did not show significant alteration in all tested genotypes. These results suggested that SA signaling pathway may mediate *ZmMEK1*^{KR}-induced early leaf senescence. We then measured SA levels in *Vector*, *ZmMEK1*^{WT}, *ZmMEK1*^{DD}and *ZmMEK1*^{KR} transgenic plants. As shown in Figure 4B, two lines of *ZmMEK1*^{KR} plants accumulated five- to eight-fold higher levels of SA than *Vector*, *ZmMEK1*^{WT}, and *ZmMEK1*^{DD} plants. The result suggested that expression of *ZmMEK1*^{KR} in *Arabidopsis* transgenic plants increases SA accumulation and enhances SA-responsive genes expression.

Early leaf senescence induced by *ZmMEK1*^{KR} dependent on SA accumulation

To further know whether elevated SA level induced by ZmMEK1^{KR} expression is required for the leaf senescence phenotype, we generated *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* plants by crossing *ZmMEK1*^{KR} with the *nahG* transgenic plant (*nahG* codes a SA-degrading salicylate hydroxylase) or with *eds5-1* mutant (*eds5-1* is defective in SA biosynthesis), respectively (Rogers and Ausubel 1997; Zhou et al. 1998). As shown in Figure 5A, when the rosette leaves of *ZmMEK1*^{KR} plants become yellow, the leaves of *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* still stay as green as the *Vector* control plants (Figure 5A). Consistently, the chlorophyll contents and membrane ion leakage in leaves of *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* plants restored to the levels in *Vector* plants (Figure 5B). As expected, the SA levels in *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* plants were significantly reduced and reached the level of *Vector* control plants (Figure 5C). These results demonstrated the phenotype of early leaf senescence caused by *ZmMEK1*^{KR} can be rescued by reducing endogenous levels of SA, suggesting that *ZmMEK1*^{KR}-induced leaf senescence is SA-dependent.

ZmMEK1 activates *Arabidopsis* MPK4

Since no obvious phenotype of early leaf senescence or SA over-production was detected in *ZmMEK1*^{WT} or *ZmMEK1*^{DD} transgenic *Arabidopsis* plants but only in *ZmMEK1*^{KR} plants, we inferred that the introduction of *ZmMEK1*^{KR} suppressed at least one *Arabidopsis* MAPK pathway which functions as a negative regulator of senescence and then results in early leaf senescence and SA accumulation. To identify which MAPK pathway(s) are blocked by ZmMEK1^{KR}, we need to determine the *Arabidopsis* MPK(s) activated by ZmMEK1. Phylogenetic analysis showed that the homolog of ZmMEK1 in *Arabidopsis* is MKK6 (Figure S3). A previous study has reported that MKK6 phosphorylated MPK4 (Kosetsu et al. 2010) which is the *Arabidopsis* ortholog of ZmSIMK1 (Guet al. 2010; Figure S3). According to the above data that ZmMEK1 interacted with ZmSIMK1 in maize protoplast, we inferred that MPK4 might be the downstream component of ZmMEK1in *Arabidopsis*. So we first tested the relationship of ZmMEK1 and MPK4 using the Y2H system and found that ZmMEK1 strongly s with MPK4, but not MPK5, MPK12, MPK13, MPK3, MPK6 (self-activation), and MPK10 in yeast (Figure 6A). Then, we performed an in-gel kinase assay using recombinant His-tagged MPK4 and Flag-tagged ZmMEK1 mutant proteins. As shown in Figure 6B, MPK4 was activated by ZmMEK1^{WT}. These results suggested that MPK4 is the downstream target MAPK of ZmMEK1 in *Arabidopsis*.

Effect of SA in dark-induced leaf senescence in maize

The result that expression of *ZmMEK1*^{KR} in transgenic *Arabidopsis* plants induced SA accumulation and subsequently

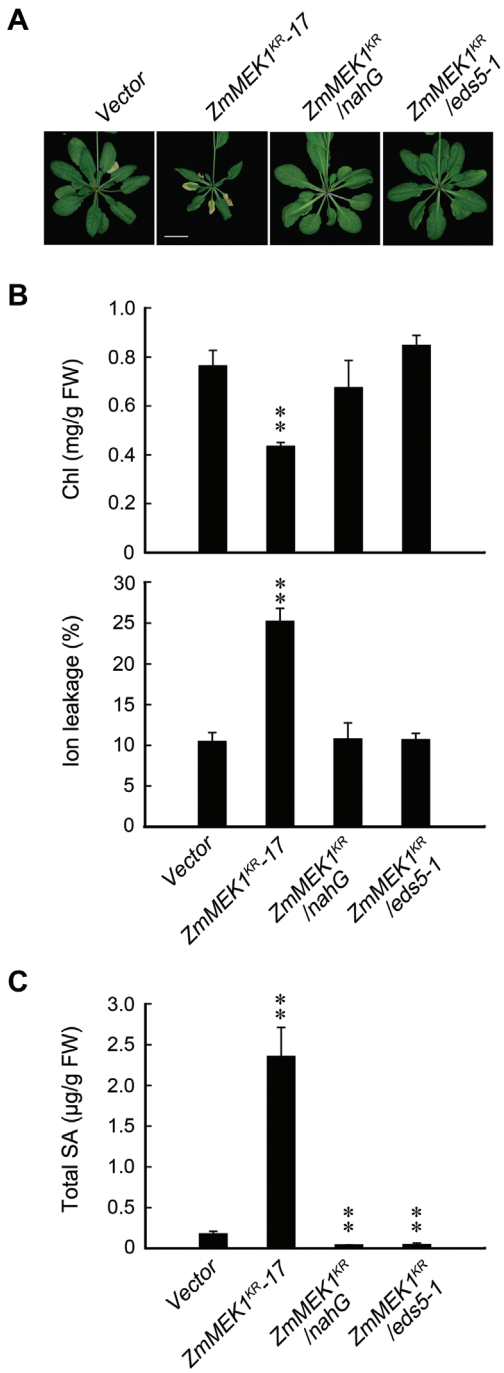

Figure 5. Early leaf senescence caused by ZmMEK1^{KR} dependent on SA accumulation

(**A**) The senescence phenotypes of 7-week-old *Vector*, *ZmMEK1*^{KR}, *ZmMEK1*^{KR}*/nahG*, and *ZmMEK1*^{KR}*/eds5-1* plants. Bar = 1 cm. (**B**) Chlorophyll (Chl) contents and membrane ion leakage, and (**C**) total SA levels in leaves of *Vector*, *ZmMEK1*^{KR}, *ZmMEK1*^{KR}*/nahG*, and *ZmMEK1*^{KR}*/eds5-1* plants. The third pair leaves of each plant grown for 7 weeks were subjected to analysis. Error bars indicate *SD* from triplicate experiments, and asterisks indicate significant differences between the *Vector*, *ZmMEK1*^{KR}, *ZmMEK1*^{KR}*/nahG*, and *ZmMEK1*^{KR}*/eds5-1* plants and *Vector* ($^{*}P < 0.05$, $^{**}P < 0.01$, *t*-test).

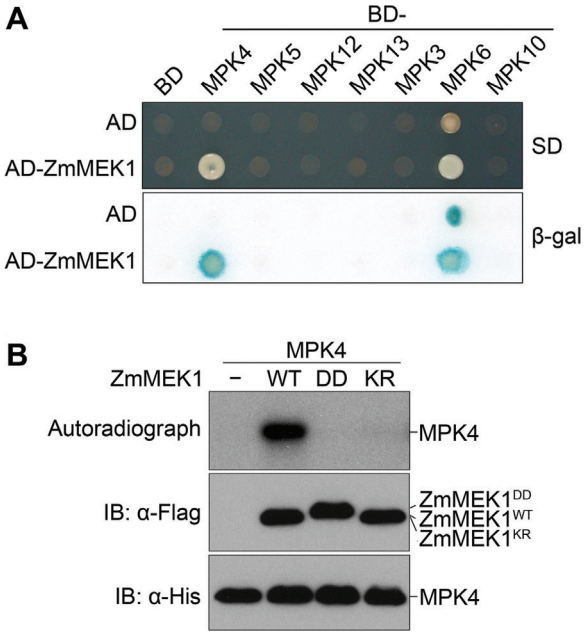

Figure 6. ZmMEK1 activates *Arabidopsis* MPK4 *in vitro*

(**A**) Interaction between ZmMEK1 and MPK4 in the yeast two-hybrid (Y2H) assay. ZmMEK1 fusions to GAL4 activating domain(AD) was coexpression with GAL4 binding domain fused MPKs in yeast cells. Interaction was examined by yeast cell growth on synthetic defined (SD) medium lacking His, Leu, Trp, and Ade (upper panel), followed by assays for β-galactosidase activity (β-gal, bottom panel). (**B**) An activation assay of *Arabidopsis* MPK4 by ZmMEK1 *in vitro*. Recombinant Flag-tagged ZmMEK1 mutant proteins were incubated with (+) or without (−) His-tagged MPK4 in a kinase reaction system. The kinase activities of MPK4 were detected by in-gel kinase assay using MBP as substrate and subjected to autoradiography (top). The protein amounts of Flag-tagged ZmMEK1 mutant proteins (middle) and His-tagged MPK4 (bottom) are shown by immunoblotting with anti-Flag and anti-His antibody, respectively.

SA-dependent leaf senescence, led us to speculate that SA may also play a role in leaf senescence in maize. We thus analyzed the effect of SA on maize IDLs. The second leaves of 2-week-old maize seedlings were injected with different concentrations of SA and then covered by aluminum foil to induce leaf senescence. After 4 d in dark, the IDLs in the absence of SA displayed symptoms of leaf senescence, such as leaves turning yellow, chlorophyll content decreasing and ion leakage increasing (Figure 7A, B, 0 mmol/L SA). With the concentration of SA increasing, the symptoms of leaf senescence in IDLs became more pronounced. In IDLs with 1.0 mmol/L SA treatment, obvious necrotic phenotype was visualized on the tip of the leaves. When the concentration of SA increased to 2.0 mmol/, the necrotic phenotype became more apparent to one-third of the treated leaves (Figure 7A). The chlorophyll contents and membrane ion leakage were consistent with the phenotype (Figure 7B). The data suggested that SA positively regulates leaf senescence in maize.

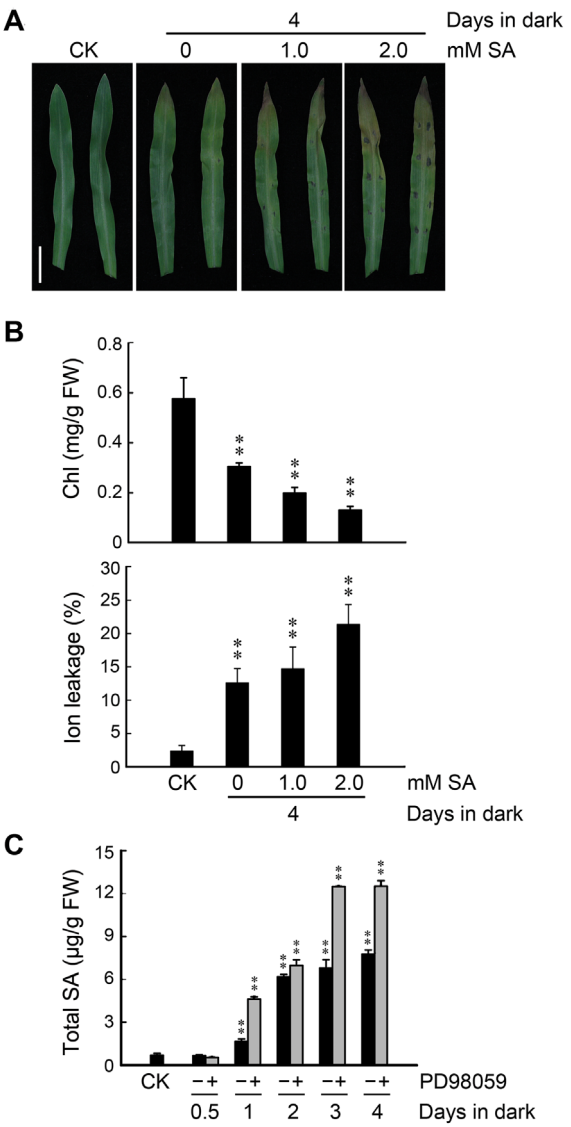

Figure 7. SA promotes dark-induced maize leaf senescence
(**A**) Effects of SA on dark-induced leaf senescence. The second leaves of 2-week-old maize seedlings were injected with indicated concentrations of SA, and then covered with aluminium foil to induce senescence for 4 d. The second leaves of 2-week-old maize seedlings without treatment were used as control (CK). Bar = 2 cm. (**B**) Chlorophyll contents and membrane ion leakage in leaves described in (**A**) were measured. Asterisks indicate significant differences between maize leaves with SA treatment and CK ($^{**}P < 0.01$, *t*-test). (**C**) Total SA levels in the indicated maize leaves. Asterisks indicate significant differences between dark-induced senescent maize leaves and CK ($^{**}P < 0.01$, *t*-test). The data in (**B**) and (**C**) are presented as the mean values of three biological replications. Error bars indicate *SD* from triplicate experiments.

Next, we determined the endogenous SA levels in maize IDLs treated with or without PD98059. As shown in Figure 7C, SA content gradually increased from 0.76 ± 0.004 μg/g fresh weight (FW) in control to 7.59 ± 0.011 μg/g FW in leaves of dark treatment at 4 d without PD98059 and to 12.84 ± 0.192 μg/g FW in IDLs at 4 d upon PD98059 treatment, indicating that the SA levels increase with the extent of senescence. In the presence of PD98059, senescent leaves accumulated more SA than leaves without PD98059 at each point, which is consistent with the phenotype that PD98059 accelerated maize leaf senescence (Figure 1A). These data suggested that senescence induces endogenous SA accumulation in maize leaves and SA participates in MAPK cascade-mediated dark-induced leaf senescence.

DISCUSSION

Leaf senescence is a critical process for seed quality determination in plants because of the relocation of biomass and energy from senescing leaves to seeds at this developmental stage. The mechanism of leaf senescence regulation and signal transduction in crop plants is important but poorly understood. Previous studies revealed that soybean receptor-like kinase GmSARK, *Arabidopsis* AtSARK, and *Arabidopsis* MKK9 positively regulate leaf senescence (Zhou et al. 2009; Xu et al. 2011). In this study, we tried to identify a certain MAPK signaling cascade that may also be involved in the regulation of maize leaf senescence. By in-gel kinase activity assay and gene transcription detection, we found that a 42 kDa MAPK was activated and *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3* gene transcriptions were strongly induced during dark-induced leaf senescence (Figure 1C, D). Although we currently did not know whether the 42 kDa MAPK is ZmSIMK1 or ZmMPK3 or other maize MAPK, the alteration of MAPK activity and transcription levels of MAPKK and MAPKs during leaf senescence suggest the involvement of MAPK cascade(s) in maize leaf senescence.

Does the MAPK cascade positively or negatively regulate leaf senescence? Due to the lack of MAPK and MAPKK loss-of-function maize mutants, we treated leaves with MAPKK activities specific inhibitor PD98059 (Beck et al. 2010; Wang et al. 2010b) and checked the senescence phenotypes. The inhibitor treatment abolished the activity of the 42 kDa MAPK, reduced transcription induction of *ZmMEK1*, *ZmSIMK1*, and *ZmMPK3*, and accelerated dark-induced leaf senescence rate (Figure 1). The results imply that the MAPKK and MAPKs may function as a cascade to negatively regulate maize leaf senescence. *ZmMEK1* was a previously cloned maize MAPKK gene, but its biological function has not been unraveled (Hardin and Wolniak 1998). In previous studies, biological functions of MAPKK genes were usually studied by inducing expression of a constitutively active mutant in transgenic plants, such as *Arabidopsis* $MKK3^{DD}$ (Takahashi et al. 2007), $MKK9^{DD}$ (Xu et al. 2008), and $MKK4^{DD}$/$MKK5^{DD}$ (Wang et al. 2007). Unfortunately, $ZmMEK1^{DD}$ and $ZmMEK1^{WT}$ expressing plants did not show an obvious phenotype (Figure 3B). We showed that $ZmMEK1^{KR}$, an inactive mutant of ZmMEK1 with ATP binding site mutated, still retained the ability of binding with the downstream MAPK (Figure 2D, E). Therefore, $ZmMEK1^{KR}$ was used as dominant-negative mutant and was overexpressed in *Arabidopsis* transgenic plants. Overexpression of $ZmMEK1^{KR}$ induced an early senescence phenotype, suggesting that ZmMEK1 mediated MAPK cascade negative regulation of leaf senescence. Furthermore, the findings that

ZmMEK1 interacts with *Arabidopsis* MPK4 (Figure 6) imply that *ZmMEK1*^{KR} may bind with MPK4 in its transgenic *Arabidopsis* plants and then interfere with MPK4-mediating signals to lead to early senescence phenotype.

In conserved MAPK cascade, downstream MAPK is phosphorylated and activated by upstream MAPKK and then transduces the signal to the downstream target, directing and maintaining the specification of signal transduction. To obtain further insight into biological function of MAPK cascades, it is important to identify the downstream MAPK of MAPKK. In our study, we also tried to identify the downstream MAPK of ZmMEK1. We first determined the interactions between ZmMEK1 and ZmSIMK1 both *in vitro* (Figure 2A) and *in vivo* (Figure 2B), and then we found that ZmMEK1 activates ZmSIMK1 *in vitro* (Figure 2D). Taken together, these data demonstrated ZmSIMK1 is a downstream MAPK of ZmMEK1. In addition, the enhanced expression of both ZmMEK1 and ZmSIMK1 in dark-induced senescing leaves (Figure 1D) implied the relations between ZmMEK1 and ZmSIMK1 in regulating maize leaf senescence. However, to explore this possibility, interaction between ZmMEK1 and ZmSIMK1 in senescing leaves and further genetic evidence are needed.

How does the MAPK cascade regulate leaf senescence? When we detect the marker genes expression, we found that *PR1* and *PR5*, marker genes of SA signaling pathway, were strongly induced in *ZmMEK1*^{KR} transgenic *Arabidopsis* plants. The result implies that *ZmMEK1*^{KR} expression-induced leaf senescence may be related to SA accumulation which is caused by SA over-production or SA catabolism reduction regulated by enzymes like SA 3-hydroxylase (Zhang et al. 2013). SA is an important plant hormone which is extensively investigated in various processes, especially in pathogen-plant interaction. In leaf senescence, SA was determined as a positive factor (Lim et al. 2007). Our study showed that SA levels in *ZmMEK1*^{KR} transgenic plants were significantly higher (Figure 4B). Significantly reduced SA and abolished early leaf senescence phenotype were found in *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* plants (Figure 5). These data are consistent with previous findings that senescent *Arabidopsis* leaves accumulate high levels of SA (Morris et al. 2000) and suggest the positive role of SA in *Arabidopsis* leaf senescence. However, whether SA plays a similar role in maize leaf senescence regulation remains unclear. Some reports described the function of SA in some cases in maize as different from that in *Arabidopsis*. For example, SA is a well-known inducer of systemic acquired resistance (SAR) in *Arabidopsis*; however, it seems to have little or no effect on pathogen resistance in maize (Balint-Kurti and Johal 2009). As such, we explored the role of SA in maize leaf senescence and found exogenous SA accelerates the rate of dark-induced maize leaf senescence (Figure 7). So we conclude that SA likely also plays a positive role in leaf senescence in maize. That higher levels of SA accumulate in PD98059-treated senescent leaves also provides a clue that SA may mediate the regulation of MAPK cascade in leaf senescence in maize (Figure 7). Although our findings demonstrated that SA mediates *ZmMEK1*^{KR}-induced leaf senescence in transgenic *Arabidopsis* plants and dark-induced leaf senescence in maize, a more detailed analysis of relations between MAPK cascade and SA in leaf senescence in maize needs to be performed.

MATERIALS AND METHODS

Plant materials and growth conditions

Maize (*Zea mays*, inbred line B73) seedlings in this study were grown in a growth chamber under a 16-h light/8-h dark photoperiod at 100 μmol/2 m/s at a temperature of 28/18 °C (day/night), and watered with Hoagland' solution (pH 6.0) every 2 d.

Arabidopsis thaliana plants were grown in soil in a growth room with a 16-h light/8-h dark photoperiod at a photon flux density of 100 μmol/2 m/s at 22 °C. Seeds were surface sterilized and sown on half-strength Murashige and Skoog (MS) medium (2.2 g/L MS salts, 1% sucrose, pH 5.8, and 8 g/L agar). After imbibing for 4 d at 4 °C, the seeds were germinated and grown on plates for 7 d and then transferred to soil. *Arabidopsis* wild-type plants, mutants and transgenic lines were all in the ecotype Col-0 background. *ZmMEK1*^{KR}/*nahG* and *ZmMEK1*^{KR}/*eds5-1* plants were generated by crossing *ZmMEK1*^{KR} plants with *nahG*and *eds5-1* plants, respectively.

For the chemical treatment experiments, maize leaves were injected with 200 μmol/L of PD98059 or indicated concentration of SA and then darkened to induce senescence. The leaves treated with the same concentration of dimethyl sulfate or methanol were used as the control.

Transgenic *Arabidopsis* plants generation

The maize *ZmMEK1* (GRMZM2G167856) complementary NDA (cDNA) was obtained by RT-PCR with specific primers and inserted into the pEGM-T easy vector. The constitutively kinase-active and -inactive mutants of *ZmMEK1* were generated by QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The genes of *ZmMEK1* and its mutants were inserted into the SpeI/XhoI sites of the pSuper1300 binary vector (Shi et al. 2012) containing a *GFP* coding sequence. The plasmids were electroporated into *Agrobacterium tumefaciens* strain C58C1, which was used to transform *Arabidopsis* by floral dip (Clough and Bent 1998). Gene-specific primers are listed in Table S1.

Induction of leaf senescence

Dark-induced leaf senescence of maize plants was performed as described by Keech et al. (2007). In brief, the fully extended second leaves of 2-week-old maize seedlings were covered by aluminum foil while the rest of the plant remained in the light. These leaves are referred to as IDLs. Leaves were darkened for 0.5, 1, 2, 3 or 4 d.

Measurements of chlorophyll content and membrane ion leakage

The third pair of rosette leaves of individual *Arabidopsis* plants and the IDLs of maize plants were taken and used for chlorophyll content and membrane ion leakage analyses. For chlorophyll extraction, the leaves were ground with 95% ethanol and incubated for 3 h. Chlorophyll content was determined at 663 and 645 nm as previously described by Lichtenthaler (1987). The membrane ion leakage was detected as previously described (Li et al. 2013).

Gene expression quantification by qRT-PCR

Total RNA was isolated from leaves of *Arabidopsis* or maize using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-

strand cDNA synthesis was performed using 1 μg of total RNA with oligo (dT) 16 as the primer and Moloney Murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocol. Real-time PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), using SYBR Green Mix (Takara, Dalian, China). Expression levels of *ubiquitin5* and 18S ribosomal RNA (rRNA) were used as the internal controls in *Arabidopsis* and maize, respectively. Primers are listed in Table S1.

Yeast two-hybrid assay

The yeast two-hybrid system (Clontech, Palo Alto, CA, USA) was used to test the interaction between ZmMEK1 and ZmMPKs or *Arabidopsis* MPKs. The coding regions of *ZmMPKs* or MPKs were amplified by PCR and inserted into the plasmid pGBKT7, which contains the GAL4 DNA binding domain, to produce the bait plasmids. The cDNA encoding *ZmMEK1* was PCR amplified and cloned into pGADT7 vector, which contains the GAL4 activation domain, creating the prey plasmid pGADT7-ZmMEK1. The bait and prey plasmids were then cotransformed into yeast strain AH109. Transformants were screened by measuring the growth of yeast cells on standard synthetic dropout medium (SD/-His/-Leu/-Trp/-Ade). The β-gal assay was performed to determine protein-protein interactions using 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside (X-gal) as substrate.

Protoplast transient expression and coimmunoprecipitation

Coding regions of *ZmMEK1* and *ZmMPKs* were amplified by PCR and cloned in frame into the 326-N-Myc and 326-N-Flag vectors (Zhao et al. 2014), respectively, resulting in the Myc-ZmMEK1 and Flag-ZmMPKs constructs under the control of the 35S promoter. Transformation of maize leaf mesophyll protoplast was performed as described (http://genetics.mgh.harvard.edu/sheenweb/). In brief, protoplasts were isolated from 11- to 13-d-old etiolated maize seedlings (inbred B73 line). The second or third leaves were chopped into small pieces and then digested in 0.6 mol/L mannitol, 10 mmol/L 2-(N-Morpholino)ethanesulfonic acid (MES), pH 5.7, 1 mmol/L $CaCl_2$, and 0.1% (w/v) bovine serum albumin (BSA) supplemented with 1.5% cellulose R10, 0.3% macerozyme R10 (Yakult Honsha Co., Tokyo, Japan) for 15 min under vacuum followed by 3 h gentle shaking (40 rpm) at 25 °C. After releasing the protoplasts at 90 rpm, the protoplasts were filtered through a 35-μm nylon mesh and collected by centrifugation at 150 × *g* for 1 min. Protoplasts were washed twice with W5 buffer (0.6 mol/L mannitol, 2 mmol/L MES, pH 5.7, and 5 mmol/L KCl, 125 mmol/L $CaCl_2$, 154 mmol/L $CaCl_2$) and then incubated on ice for 30 min. After collection by centrifugation at 150 × *g* for 1 min, protoplasts were resuspended with MMg solution (0.4 mol/L mannitol, 15 mmol/L $MgCl_2$, and 4 mmol/L MES, pH 5.7). Ten micrograms of plasmid 326-Myc-ZmMEK1 and 326-Flag-ZmMPKs plasmids, respectively, were transfected to 200 μL protoplasts (4×10^4 cells). Polyethylene glycol (PEG) solution (220 μL 40% PEG 4000 (w/v), 0.2 mol/L mannitol, and 0.1 mol/L $CaCl_2$) was added to protoplasts by gently inverting the tubes. Protoplasts were incubated in the dark for 30 min at room temperature and then 800 μL W5 solution was added to terminate transfection. After harvesting by centrifugation at 150 × *g* for 2 min, transfected protoplasts were resuspended with 100 μL W5 solution and then incubated in the dark for 12–16 h at 25 °C to allow protein expression.

For coimmunoprecipitation assays, protoplasts with expression constructs were lysed by vigorous shaking in immunoprecipitation buffer (20 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L ethyleneglycoltetraacetic acid, 1 mmol/L NaF, 1 mmol/L $NaVO_3$, 10 mmol/L β-glycerophosphate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1% (v/v) Triton X-100). Anti-MycAffinity Agrose Gel (Sigma–Aldrich, St Louis, MO, USA) was used for immunoprecipitation. After washing three times with the TBS buffer (20 mmol/L Tris-HCl, pH 7.5 and 150 mmol/L NaCl), immunoprecipitates were subjected to immunoblot analysis with anti-Flag antibody.

Immunoblot assay

Protein extraction, separation and immunoblot analyses were performed as previously described (Su et al. 2011). Monoclonal anti-Flag antibody, monoclonal anti-His antibody, monoclonal anti-Myc antibody (Sigma-Aldrich) were used as primary antibodies, and a horseradish peroxidase-conjugated goat anti-mouse antibody was used as secondary antibody in this study. The signal on immunoblot was detected by an enhanced chemiluminescence kit and exposed to X-ray film.

Pull-down assays

For the pull-down assay, 5 μg of His-tagged ZmSIMK1 or GFP protein was incubated with 5 μg of Flag-tagged ZmMEK1 mutant proteins, respectively, at 25 °C for 30 min in pull-down buffer (20 mmol/L HEPEs-NaOH, pH 7.5, 10 mmol/L $MnCl_2$, 10 mmol/L $MgCl_2$, and 50 μmol/L ATP). Then, the Chelating Sepharose Fast Flow (GE Healthcare, Piscataway, NJ, USA) affinited nickel (Ni^{2+}) ion was used to pull down ZmSIMK1. After washing three times with washing buffer (20 mmol/L HEPES-NaOH, pH 7.5, 300 mmol/L NaCl), the resin was added with 20 μL of sodium dodecyl sulfate (SDS) loading buffer. Samples were analyzed by immunoblotting using anti-His or anti-Flag antibody.

Autophosphorylation assay

The *in vitro* autophosphorylation assay was performed according to a previously described method (Xu et al. 2008). Equal amounts (2 μg) of purified recombinant ZmMEK1 and its mutants were incubated at 30 °C for 30 min in a total volume of 30 μL of reaction mixture containing 10 mmol/L HEPEs (pH 7.5), 1 mmol/L $MgCl_2$ or $MnCl_2$, 1 mmol dithiothreitol, and 1 μCi [γ-^{32}P] ATP. The reaction was stopped by the addition of SDS loading buffer and then subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and autoradiography.

Protein preparation and in-gel kinase assays

Total protein of plant samples was extracted and subjected to in-gel kinase assay as previously descried (Xu et al. 2008). For recombinant protein kinase analysis, 2 μg of His-tagged MPK proteins purified on Chelating Sepharose Fast Flow was activated by 0.2 μg of Flag-tagged ZmMEK1 mutant proteins purified using ANTI-Flag M2 Affinity Gel (Sigma–Aldrich) at 25 °C for 30 min in 20 mmol/L HEPES-NaOH (pH 7.5), 10 mmol/L $MnCl_2$, 10 mmol/L $MgCl_2$, and 50 μmol/L ATP in a final volume of 30 μL. The reaction was stopped by addition of 10 μL of 4 × SDS loading buffer and the kinase activities were

determined by an in-gel kinase assay. Myelin basic protein (MBP) was used as the substrate.

SA extraction and measurement

Rosette leaves of 5-week-old *Arabidopsis* plants grown on soil were harvested; 300–500 mg of samples were extracted twice with 5 mL of 95% methanol for 1 h, dried, and resuspended in 500 μL of 5% trichloroacetic acid. The samples were further extracted with 1 mL of extraction solvent cyclohexane/ethyl acetate (1:1, v/v). The top phase which contains free SA was collected. The bottom aqueous phase which contains conjugated SA was added to HCl to a final concentration of 6 mol/L to hydrolyze amino acid and then extraction with 1 mL of extraction solvent. The SA released from conjugated SA was in the top phase. Combined the twice top phase and dried, and total SA was in it. Total SA was dissolved in 200 μL of 10% methonal in 20 mmol/L sodium acetate buffer, pH 5.5, and then subjected to high-performance liquid chromatography (HPLC) analysis. SA analysis was performed using the 1260 Infinity HPLC (Aligent Technology, Wilmington, DE, USA) with a Luna 5u C18 100A analytic column (250 × 4.6 mm, Phenomenex, Torrance, CA, USA) run with 10% methonal in 20 mmol/L sodium acetate buffer, pH 5.5, at a flow rate of 1 mL/min for 30 min. SA was detected and fluorometrically quantified (excitation 294 nm; emission 426 nm).

ACKNOWLEDGEMENTS

This work was supported by grants from the State Basic Research Program (2014CB138205) and the National Natural Science Foundation of China (31125006 and 31030010) to D. Ren; the National Natural Science Foundation of China (30771124) to H. Yang; and the National Natural Science Foundation of China (31000127) to Y. Li.

AUTHOR CONTRIBUTIONS

Y.L. and D.T.R. designed research; Y.L., Y.C., C.C.Z., H.L.Y. performed research; Y.L. and D.T.R. analyzed data; and Y.L. and D.T.R. wrote the paper.

REFERENCES

Balint-Kurti PJ, Johal GS (2009) Maize disease resistance. In: Bennetzen JL, Hake SC, eds. *Handbook of Maize: Its Biology*. Springer, New York. pp. 229–253

Beck M, Komis G, Ziemann A, Menzel D, Šamaj J (2010) Mitogen-activated protein kinase 4 is involved in the regulation of mitotic and cytokinetic microtubule transitions in *Arabidopsis thaliana*. **New Phytol** 189: 1069–1083

Berberich T, Sano H, Kusano T (1999) Involvement of a MAP kinase, ZmMPK5, in senescence and recovery from low-temperature stress in maize. **Mol Gener Genet** 262: 534–542

Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D, Zhang C, Morris K, Jenner C, Jackson S, Thomas B, Tabrett A, Legaie R, Moore JD, Wild DL, Ott S, Rand D, Beynon J, Denby K, Mead A, Buchanan-Wollaston V (2011) High-resolution temporal profiling of transcripts during *Arabidopsis* leaf senescence reveals a distinct chronology of processes and regulation. **Plant Cell** 23: 873–894

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. **Plant J** 42: 567–585

Cai GH, Wang GD, Wang L, Pan JW, Liu Y, Li DQ (2014) *ZmMKK1*, a novel group A mitogen-activated protein kinase kinase gene in maize, conferred chilling stress tolerance and was involved in pathogen defense in transgenic tobacco. **Plant Sci** 214: 57–73

Cardinale F, Meskiene I, Ouaked F, Hirt H (2002) Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. **Plant Cell** 14: 703–711

Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. **Plant J** 16: 735–743

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. **Proc Natl Acad Sci USA** 92: 7686–7689

Gregersen PL, Culetic A, Boschian L, Krupinska K (2013) Plant senescence and crop productivity. **Plant Mol Biol** 82: 603–622

Gu L, Liu Y, Zong X, Liu L, Li DP, Li DQ (2010) Overexpression of maize mitogen-activated protein kinase gene, *ZmSIMK1* in *Arabidopsis* increases tolerance to salt stress. **Mol Biol Rep** 37: 4067–4073

Guiboileau A, Sormani R, Meyer C, Masclaux-Daubresse C (2010) Senescence and death of plant organs: Nutrient recycling and developmental regulation. **CR Biol** 333: 382–391

Guo YF, Cai ZH, Gan SS (2004) Transcriptome of *Arabidopsis* leaf senescence. **Plant Cell Environ** 27: 521–549

Guo YF, Gan SS (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. **Plant J** 46: 601–612

Guo YF, Gan SS (2012) Convergence and divergence in gene expression profiles induced by leaf senescence and 27 senescence-promoting hormonal, pathological and environmental stress treatments. **Plant Cell Environ** 35: 644–655

Guo YF, Gan SS (2011) *AtMYB2* regulates whole plant senescence by inhibiting cytokinin-mediated branching at late stages of development in *Arabidopsis*. **Plant Physiol** 156: 1612–1619

Hardin SC, Wolniak SM (1998) Molecular cloning and characterization of maize ZmMEK1, a protein kinase with a catalytic domain homologous to mitogen- and stress-activated protein kinase kinases. **Planta** 206: 577–584

Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champion A, Kreis M, Zhang SQ, Hirt H, Wilson C, Heberle-Bors E, Ellis BE, Morris PC, Innes RW, Ecker JR, Scheel D, Klessig DF, Machida Y, Mundy J, Ohashi Y, Walker JC, Grp M (2002) Mitogen-activated protein kinase cascades in plants: A new nomenclature. **Trends Plant Sci** 7: 301–308

Jiang YJ, Liang G, Yang SZ, Yu DQ (2014) *Arabidopsis* WRKY57 functions as a node of convergence for jasmonic acid- and auxin-mediated signaling in jasmonic scid-induced leaf senescence. **Plant Cell** 26: 230–245

Jibran R, Hunter D, Dijkwel P (2013) Hormonal regulation of leaf senescence through integration of developmental and stress signals. **Plant Mol Biol** 82: 547–561

Keech O, Pesquet E, Ahad A, Askne A, Nordvall D, Vodnala SM, Tuominen H, Hurry V, Dizengremel P, Gardestrom P (2007) The different fates of mitochondria and chloroplasts during dark-

induced senescence in *Arabidopsis* leaves. **Plant Cell Environ** 30: 1523–1534

Kiegerl S, Cardinale F, Siligan C, Gross A, Baudouin E, Liwosz A, Eklöf S, Till S, Bögre L, Hirt H, Meskiene I (2000) SIMKK, a Mitogen-Activated Protein Kinase (MAPK) Kinase, is a specific activator of the salt stress-induced MAPK, SIMK. **Plant Cell** 12: 2247–2258

Kong XP, Pan JW, Zhang MY, Xing X, Zhou Y, Liu Y, Li DP, Li DQ (2011) *ZmMKK4*, a novel group C mitogen-activated protein kinase kinase in maize (*Zea mays*), confers salt and cold tolerance in transgenic *Arabidopsis*. **Plant Cell Environ** 34: 1291–1303

Kosetsu K, Matsunaga S, Nakagami H, Colcombet J, Sasabe M, Soyano T, Takahashi Y, Hirt H, Machida Y (2010) The MAP kinase MPK4 is required for cytokinesis in *Arabidopsis thaliana*. **Plant Cell** 22: 3778–3790

Li LB, Li Y, Zhang LH, Xu CH, Su TB, Ren DT, Yang HL (2012a) Sucrose induces rapid activation of CfSAPK, a mitogen-activated protein kinase, in *Cephalostachyum fuchsianum* Gamble cells. **Plant Cell Environ** 35: 1428–1439

Li ZH, Peng JY, Wen X, Guo HW (2012b) Gene network analysis and functional studies of senescence-associated genes reveal novel regulators of *Arabidopsis* leaf senescence. **J Integr Plant Biol** 54: 526–539

Li ZH, Peng JY, Wen X, Guo HW (2013) *Ethylene-insensitive3* is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing *miR164* transcription in *Arabidopsis*. **Plant Cell** 25: 3311–3328

Lichtenthaler HK (1987) Chlorophylls and carotenoids-pigments of photosynthetic biomembranes. **Methods Enzymol** 148: 350–382

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. **Annu Rev Plant Biol** 58: 115–136

Lin JF, Wu SH (2004) Molecular events in senescing *Arabidopsis* leaves. **Plant J** 39: 612–628

Liu HX, Wang Y, Xu J, Su TB, Liu GQ, Ren DT (2008) Ethylene signaling is required for the acceleration of cell death induced by the activation of AtMEK5 in *Arabidopsis*. **Cell Res** 18: 422–432

Miao Y, Laun T, Zimmermann P, Zentgraf U (2004) Targets of the WRKY53 transcription factor and its role during leaf senescence in *Arabidopsis*. **Plant Mol Biol** 55: 853–867

Morris K, Mackerness SAH, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. **Plant J** 23: 677–685

Pan JW, Zhang MY, Kong XP, Xing X, Liu Y, Zhou Y, Liu Y, Sun LP, Li DQ (2012) *ZmMPK17*, a novel maize group D MAP kinase gene, is involved in multiple stress responses. **Planta** 235: 661–676

Pillitteri LJ, Dong J (2013) Stomatal development in *Arabidopsis*. **Arabidopsis Book** 11: e0162. doi: 10.1199/tab.0162

Ren DT, Liu YD, Yang KY, Han L, Mao GH, Glazebrook J, Zhang SQ (2008) A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. **Proc Natl Acad Sci USA** 105: 5638–5643

Ren DT, Yang HP, Zhang SQ (2002) Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. **J Biol Chem** 277: 559–565

Rodriguez MC, Petersen M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. **Annu Rev Plant Biol** 61: 621–649

Rogers EE, Ausubel FM (1997) *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. **Plant Cell** 9: 305–316

Shi YT, Tian SW, Hou LY, Huang XZ, Zhang XY, Guo HW, Yang SH (2012) Ethylene signaling negatively regulates freezing tolerance by repressing expression of *CBF* and type-A *ARR* genes in *Arabidopsis*. **Plant Cell** 24: 2578–2595

Soyano T, Nishihama R, Morikiyo K, Ishikawa M, Machida Y (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. **Genes Dev** 17: 1055–1067

Takahashi F, Yoshida R, Ichimura K, Mizoguchi T, Seo S, Yonezawa M, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2007) The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. **Plant Cell** 19: 805–818

Thomas H (2013) Senescence, ageing and death of the whole plant. **New Phytol** 197: 696–711

Wang HC, Ngwenyama N, Liu Y, Walker JC, Zhang SQ (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. **Plant Cell** 19: 63–73

Wang JX, Ding HD, Zhang AY, Ma FF, Cao JM, Jiang MY (2010a) A novel mitogen-activated protein kinase gene in maize (*Zea mays*), *ZmMPK3*, is involved in response to diverse environmental cues. **J Integr Plant Biol** 52: 442–452

Wang PC, Du YY, Li Y, Ren DT, Song CP (2010b) Hydrogen peroxide-mediated activation of MAP kinase 6 modulates nitric oxide biosynthesis and signal transduction in *Arabidopsis*. **Plant Cell** 22: 2981–2998

Weaver LM, Amasino RM (2001) Senescence is induced in individually darkened *Arabidopsis* leaves but inhibited in whole darkened plants. **Plant Physiol** 127: 876–886

Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munne-Bosch S, Antonio C, Tohge T, Fernie AR, Kaufmann K, Xue GP, Mueller-Roeber B, Balazadeh S (2012) JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in *Arabidopsis*. **Plant Cell** 24: 482–506

Xu F, Meng T, Li PL, Yu YQ, Cui YJ, Wang YX, Gong QQ, Wang NN (2011) A soybean dual-specificity kinase, GmSARK, and its *Arabidopsis* homolog, AtSARK, regulate leaf senescence through synergistic actions of auxin and ethylene. **Plant Physiol** 157: 2131–2153

Xu J, Li Y, Wang Y, Liu HX, Lei L, Yang HL, Liu GQ, Ren DT (2008) Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in *Arabidopsis*. **J Biol Chem** 283: 26996–27006

Xu J, Xie J, Yan CF, Zou XQ, Ren DT, Zhang SQ (2014) A chemical genetic approach demonstrates that MPK3/MPK6 activation and NADPH oxidase-mediated oxidative burst are two independent signaling events in plant immunity. **Plant J** 77: 222–234

Zhang KW, Halitschke R, Yin CX, Liu CJ, Gan SS (2013) Salicylic acid 3-hydroxylase regulates *Arabidopsis* leaf longevity by mediating salicylic acid catabolism. **Proc Natl Acad Sci USA** 110: 14807–14812

Zhang MY, Pan JW, Kong XP, Zhou Y, Liu Y, Sun LP, Li DQ (2012) *ZmMKK3*, a novel maize group B mitogen-activated protein kinase kinase gene, mediates osmotic stress and ABA signal responses. **J Plant Physiol** 169: 1501–1510

Zhao Y et al. (2014) REPRESSOR OF SILENCING5 encodes a member of the small heat shock protein family and is required for DNA demethylation in *Arabidopsis*. **Plant Cell Online** 26: 2660–2675

Zhou CJ, Cai ZH, Guo YF, Gan SS (2009) An *Arabidopsis* mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. **Plant Physiol** 150: 167–177

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. **Plant Cell** 10: 1021–1030

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Transcript levels of *ZmMKKs* and *ZmMPKs* in dark-induced senescing leaves

(**A**) Expression of *ZmMKK1*, *ZmMKK3*, and *ZmMKK4*. (**B**) Expression of *ZmMPK4*, *ZmMPK5*, *ZmMPK6*, *ZmMPK7*, and *ZmMPK17*. The second leaves of 2-week-old maize seedlings were covered with aluminium foil to induce senescence for 0.5, 1, 2, 3 or 4 d with (+) or without (−) 200 μmol/L of PD98059. The second leaves of 2-week-old maize seedlings without treatment were used as a control (CK). Total RNA was extracted from leaves of plants after dark treatment and applied to quantitative real-time reverse transcription polymerase chain reaction analysis. Expression levels were normalized to the transcript levels of the control leaves. Error bars indicate *SD* from triplicate experiments.

Figure S2. Immunoblot analysis of ZmMEK1 proteins in transgenic *Arabidopsis* plants

Total proteins were extracted from the third pair rosette leaves of *Arabidopsis* plants expressing green fluorescent protein (GFP) (Vector) and GFP-fused ZmMEK1 mutants ($ZmMEK1^{WT}$, $ZmMEK1^{DD}$, and $ZmMEK1^{KR}$), respectively, and subjected to immunoblot analysis with anti-GFP antibody (top and middle panel). Two independent transgenic lines of *ZmMEK1* were used for analysis. Each line was loaded with 10 μg total protein. The result of immunoblot with anti-tubulin antibody was used as the loading control (bottom panel).

Figure S3. Phylogenetic relationships of ZmMEK1 protein with *Arabidopsis* MKKs (**A**) and ZmSIMK1 with *Arabidopsis* MPKs (**B**) Amino acid sequences were aligned using the Lasergene Megalign program (DNAstar)

Table S1. Primers used in this study